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ORIGINAL ARTICLE

Serum matrix metalloproteinase 8 and tissue inhibitor of metalloproteinase 1: Potential markers for malignant transformation of recurrent respiratory papillomatosis and for prognosis of laryngeal cancer

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Abstract

Background: Biomarkers that could predict malignant transformation of recurrent respiratory papillomatosis (RRP) would be useful in patient follow-up. We investigated whether serum matrix metalloproteinase 8 (MMP-8) and tissue inhibitor of metalloproteinase 1 (TIMP-1) could predict malignant transformation of RRP and whether they associate with survival in laryngeal squamous cell carcinoma (LSCC) without preexisting RRP.

Methods: We analyzed serum MMP-8 (S-MMP-8) and serum TIMP-1 (s-TIMP-1) in 114 patients: 55 were treated for RRP and 59 for LSCC without preexisting RRP. Five patients with RRP developed LSCC during follow-up.

Results: Elevated S-MMP-8 level in RRP was associated with malignant transformation ($P = .01$). Compared to patients with RRP, S-MMP-8 in patients with LSCC was significantly higher ($P < .001$). Increased S-TIMP-1 level in LSCC was associated with poor overall survival ($P = .02$) and recurrence-free survival ($P = .05$).

Conclusion: In RRP, high S-MMP-8 may predict malignant transformation. In LSCC, elevated S-TIMP-1 is connected to poor survival.

KEYWORDS

biomarkers, laryngeal carcinoma, MMP-8, recurrent respiratory papilloma, TIMP-1

1 | INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is a disease characterized by recurrent benign epithelial tumors that typically present in the larynx. The main causative agents are low-risk human papillomavirus (HPV) types 6 and 11.

Matrix metalloproteinases (MMPs) are genetically distinct but structurally related proteolytic enzymes, which can modulate the degradation of extracellular matrix components and basement membranes. They also modulate inflammatory responses by processing various nonmatrix bioactive subsites such as growth factors, pro-inflammatory and antiinflammatory cytokines. MMP-8 degrades collagen type 1. It is also associated with many inflammatory conditions. Nurmenniemi et al. have shown that in patients with head and neck squamous cell carcinoma, elevated plasma MMP-8 levels are associated with stage IV disease.[1] Tissue inhibitors of metalloproteinases (TIMPs) regulate the activity of MMPs, and in addition they function as growth factors.[2]

Part of this study was presented in abstract form at 4th Congress of European ORL-HNS 7-11 October 2017; Barcelona, Spain.

Overall survival (OS) in patients with head and neck cancer with enhanced systemic TIMP-1 response is poor.[1,3,4] However, the survival rates have not been reported independently by primary tumor site.

Our previous study combined data of patients with RRP from the Helsinki University Hospital database and the Finnish Cancer Registry. We showed that 9 of 324 (2.8%) patients with RRP, treated between 1975 and 2011, developed laryngeal squamous cell carcinoma (LSCC).[5] The aim of this study was to further analyze this patient group and to assess whether S-MMP-8 and S-TIMP-1 could predict malignant transformation of RRP. Moreover, we compared S-MMP-8 and S-TIMP 1 levels of patients with RRP and LSCC and analyzed whether the serum levels in patients with LSCC were associated with survival.

2 | PATIENTS AND METHODS

2.1 | Patients

2.1.1 | Recurrent respiratory papillomatosis

We included all patients with RRP who had donated serum between 1996 and 2004 for the Helsinki University Hospital Head and Neck Tumor Bank and were aged 40 years or older when the serum samples were drawn. National Cancer Registry data confirmed all malignancies diagnosed before the year 2012. Of the 55 patients (median age 55 years, range 40-77, 76% males), 5 developed LSCC (Table 1). Formalin-fixed paraffin-embedded laryngeal tissue blocks from patients with RRP who developed LSCC were retrieved from the archives of the Department Pathology and processed for standard hematoxylin and eosin–stained sections. The study pathologist (J.K.H.) carefully assessed the stained sections, and papilloma histology with koilocytes, parakeratosis/hyperkeratosis, and papillomatous epithelium with a fibrovascular core was confirmed using stringent criteria.[6] Between January 2012 and October 2017, 16 of the 55 patients (29%) had undergone follow-up examination, but no additional malignancies were diagnosed.

TABLE 1 Clinical characteristics of 5 male patients with RRP transforming into LSCC

Characteristics	Values
No. of male patients (%)	5 (100)
Age (y) at RRP diagnosis, median (range)	60 (4-73)
No. of laryngeal procedures before LSCC, median (range)	6 (3-18)
Duration (y) of RRP before LSCC, median (range)	7 (3-63)
Time (y) from obtaining the serum sample until LSCC, median (range)	5 (0.7-6.6)
Age (y) at LSCC diagnosis, mean (range)	68 (57-80)
T classification, no. of patients	
Tis	1
T1	3
T2	0
T3	1
T4	0

Abbreviations: LSCC, laryngeal squamous cell carcinoma; RRP, recurrent respiratory papillomatosis; Tis, tumor in situ.

2.1.2 | LSCC without preexisting RRP

To analyze whether S-MMP-8 and S-TIMP-1 levels were similar in RRP and LSCC, and to evaluate association between serum values and LSCC survival, we included 59 patients with LSCC (median age 66 years, range 26-88) without preexisting RRP (Table 2). These patients had donated serum for the Helsinki University Hospital Head and Neck Tumor Bank at the time of diagnosis. All study patients were treated with curative intent. T1 tumors were primarily treated with a single modality, either surgery or radiotherapy. Of the 29 patients with T2-T3 tumors, 19 (66%) were treated with chemoradiotherapy, 5 (17%) with radiotherapy, 4 (14%) with surgery followed by radiotherapy or chemoradiation, and 1 (3%) with surgery only. Of the 11 patients with T4 tumors, 8 (73%) underwent total laryngectomy followed by either radiotherapy or chemoradiation, whereas 3 (27%) underwent chemoradiotherapy only. Dates of death were provided by the Finnish Population Registry Center.

Radiotherapy was conventional external beam radiation therapy or intensity-modulated radiation therapy. Chemotherapeutic regimens were platinum-based agents given concomitantly with radiotherapy. Patients with distant metastases and those scheduled for palliative treatment at the time of presentation were excluded.

OS was defined as the duration from diagnosis to death from any cause, recurrence-free survival (RFS) from diagnosis to first documented recurrence, and disease-specific survival (DSS) from diagnosis to death caused by LSCC.

The Ethics Committee of Surgery in the Hospital District of Helsinki and Uusimaa approved the study, and all patients gave their informed written consent. Institutional permission was granted.

2.2 | Serum MMP-8 and TIMP-1 analyses

Serum samples were centrifugated, and the supernatants were stored at −70°C. Time-resolved immunofluorometric assay was used to determine S-MMP-8 concentrations, as described earlier.[7,8] A commercially available enzyme-linked immune sorbent assay (Amersham Biosciences UK Ltd, Buckinghamshire, United Kingdom) was used to determine S-TIMP-1 concentrations. The detection limit was 1.25 ng/mL for TIMP-1 and 0.8 ng/mL for MMP-8. The levels of MMP-8 and TIMP-1 were expressed as nanograms per milliliter; for calculation of MMP-8/TIMP-1 molar ratios, the levels were converted to moles per liter.

2.3 | Statistical analyses

SPSS Statistics 20.0 (IBM, Armonk, New York) was used for statistical analyses. Mann-Whitney *U* test was used to

TABLE 2 Clinical characteristics of 59 patients with LSCC without preexisting RRP

Characteristics	Values
No. of male patients (%)	50 (85)
Age (y) at LSCC diagnosis, median (range)	66 (26–88)
Smoking, no. of patients (%)	
Yes	53 (90)
No	4 (7)
NA	2 (3)
Subsite, no. of patients (%)	
Glottic or several subsites	51 (86)
Supraglottic	7 (12)
Subglottic	1 (2)
Histological grade, no. of patients (%)	
Grade 1	13 (22)
Grade 2	26 (44)
Grade 3	7 (12)
Anaplastic	1 (2)
NA	12 (20)
T classification, no. of patients (%)	
T1	19 (32)
T2	14 (24)
T3	15 (25)
T4	11 (19)
Stage, no. of patients (%)	
I-II	31 (53)
III-IV	28 (47)
Primary treatment, no. of patients (%)	
Surgery only	13 (22)
Radiotherapy only	12 (20)
Chemoradiotherapy only	22 (37)
Surgery + radiotherapy	9 (15)
Surgery + chemoradiotherapy	3 (5)

Abbreviations: LSCC, laryngeal squamous cell carcinoma; NA, not available; RRP, recurrent respiratory papillomatosis.

compare serum concentrations between any 2 groups. Pearson correlation test was used to analyze correlations between serum values and age. Kaplan-Meier method and log rank test served to compare association between serum values and survival in patients with LSCC. Patients with LSCC were classified according to their serum concentrations, so that the highest quintile, 3 middle quintiles, and the lowest quintile comprised 3 groups for comparison. Cox regression was used to analyze whether $\log_{10}$ transformed serum values, adjusted for age, sex, and stage (I-II vs III-IV), were independently associated with survival. Statistically significant P value was set at .05.

3 | RESULTS

In total, this study comprised 114 patients of whom 55 were primarily treated for RRP and 59 for LSCC without preexisting RRP. Clinical characteristics of 5 patients with RRP who developed LSCC are presented in Table 1, and those of the 59 patients treated for LSCC without preexisting RRP are presented in Table 2. Of the 59 patients with LSCC, 30 (51%) survived, and their mean duration of follow-up was 6.7 years (range 0.9-10.8 years). Of the 59 patients, 13 (22%) developed a disease recurrence. Five of the 13 recurrences were successfully treated with curative intent, either surgery (n = 4) or radiotherapy (n = 1), whereas 8 of the 13 had either distant metastases or locally advanced nonoperable disease. Five-year OS in patients with LSCC without preexisting RRP was 73%, RFS was 83%, and DSS was 88%.

3.1 | Serum MMP-8 and TIMP-1 in RRP and LSCC

Compared to patients with RRP, those treated for LSCC without preexisting RRP were seen with significantly higher S-MMP-8 (mean 35.0 vs 116.1 ng/mL, $P < .001$) but the differences in S-TIMP-1 were nonsignificant (141.3 vs 153.8 ng/mL, $P = .50$). Patients with LSCC also had significantly higher MMP-8/TIMP-1 molar ratio than patients with RRP (0.40 vs 0.11, $P < .001$). The comparison of serum values is presented in Table 3. Correlation between the serum values and age was insignificant in both RRP and LSCC.

TABLE 3 S-MMP-8 and S-TIMP-1 levels and MMP-8/TIMP-1 molar ratios among study groups

Patient group	S-MMP-8 (ng/mL), mean (range)	*P*-value	S-TIMP-1 (ng/mL), mean (range)	*P*-value	MMP-8/TIMP-1 molar ratio, mean (range)	*P*-value
All study subjects (N = 114)						
RRP (n = 55)	35.0 (7.2-115.6)	<.001	141.3 (72.6-195.1)	.50	0.11 (0.02-0.38)	<.001
LSCC (n = 59)	116.1 (9.1-454.7)		153.8 (48.9-391.8)		0.40 (0.03-2.71)	
RRP (n = 55)						
RRP without malignant transformation (n = 50)	31.5 (7.2-82.8)	.01	142.5 (72.6-195.2)	.23	0.10 (0.02-0.30)	.009
RRP with malignant transformation (n = 5)	70.1 (23.5-115.6)		129.4 (101.6-154.3)		0.23 (0.08-0.38)	
LSCC (n = 59)						
Stage I-II (n = 31)	91.7 (9.1–454.7)	.01	150.8 (48.9–391.8)	.63	0.35 (0.03-2.71)	.03
Stage III-IV (n = 28)	143.1 (18.7-363.2)		157.1 (80.1-301.1)		0.46 (0.05-1.23)	

Abbreviations: LSCC, laryngeal squamous cell carcinoma; RRP, recurrent respiratory papillomatosis; S-MMP-8, serum matrix metalloproteinase 8; S-TIMP-1, serum tissue inhibitor of metalloproteinase 1.

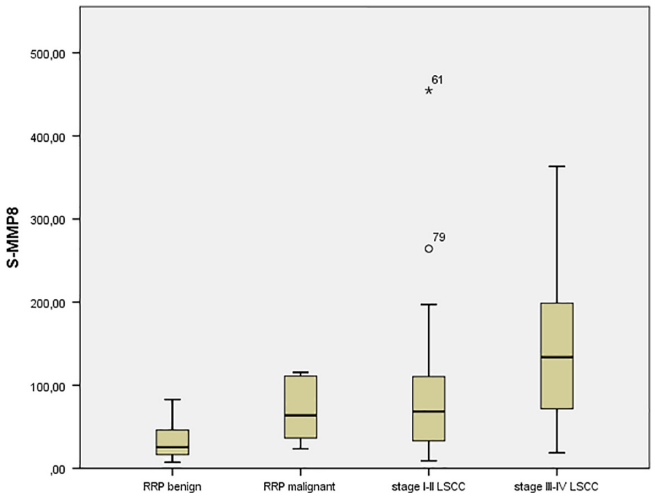

FIGURE 1 Comparison of serum matrix metalloproteinase 8 (S-MMP-8) in recurrent patients with respiratory papillomatosis (RRP) without malignancy, in patients with RRP who developed laryngeal squamous cell carcinoma (LSCC), and in patients with LSCC according to disease stage [Color figure can be viewed at wileyonlinelibrary.com]

Interestingly, even patients with LSCC with T1 tumors (n = 19) were seen with significantly higher S-MMP-8 (94.9 vs 35.0 ng/mL, $P = .002$) and MMP-8/TIMP-1 molar ratio (0.40 vs 0.11, $P = .01$) than patients with RRP.

3.2 | Serum MMP-8 and TIMP-1 in RRP with malignant transformation

Patients with RRP who later developed LSCC had significantly higher S-MMP-8 levels than those without malignant transformation (70.1 vs 31.5 ng/mL, $P = .01$). The difference was not significant for S-TIMP-1 (129.4 vs 142.5 ng/mL, $P = .23$). Mean MMP-8/TIMP-1 molar ratio was 0.10 among patients with RRP without malignant transformation and 0.23 with malignant transformation ($P = .009$). Patients with RRP who later developed LSCC were not significantly older than patients with RRP without malignancy (median age 62 vs 54 years, $P = .05$) when the serum samples were drawn. Figure 1 presents the comparison of S-MMP-8 in RRP and LSCC.

TABLE 4 Cox regression analyses for overall survival in patients with LSCC

Covariates	HR	95% CI	*P*-value
Age	1.05	1.01-1.10	.02
Sex	1.61	0.55-4.69	.38
Stage	2.28	0.94-5.54	.06
Log_{10}MMP-8	1.32	0.75-2.34	.34
Log_{10}TIMP-1	5.24	1.27-21.60	.02

Abbreviations: CI, confidence interval; HR, hazard ratio; log_{10}MMP-8, log_{10} transformed serum matrix metalloproteinase 8; log_{10}TIMP-1, log_{10} transformed serum tissue inhibitor of metalloproteinase 1; LSCC, laryngeal squamous cell carcinoma.

3.3 | Serum MMP-8 and TIMP-1 without preexisting RRP

Compared to patients with stage I-II LSCC, those seen with stage III-IV disease had significantly higher S-MMP-8 (91.7 vs 143.1 ng/mL, $P = .01$) and MMP-8/TIMP-1 molar ratio (0.35 vs 0.46, $P = .03$). S-TIMP-1 was not associated with stage (150.8 vs 157.1 ng/mL, $P = .63$).

Univariate Kaplan-Meier analysis with log-rank test showed that in LSCC without preexisting RRP, both S-MMP-8 ($P = .004$) and S-TIMP-1 ($P = .04$) were significantly associated with OS. Thus, in those patients seen with the highest serum values, the prognosis was poor (Figure 2).

Multivariate analysis, adjusted for age, sex, and stage (I-II vs III-IV), showed that log_{10} transformed values of S-TIMP-1 were significantly associated with OS ($P = .02$) and RFS ($P = .05$, Tables 4 and 5). Association between

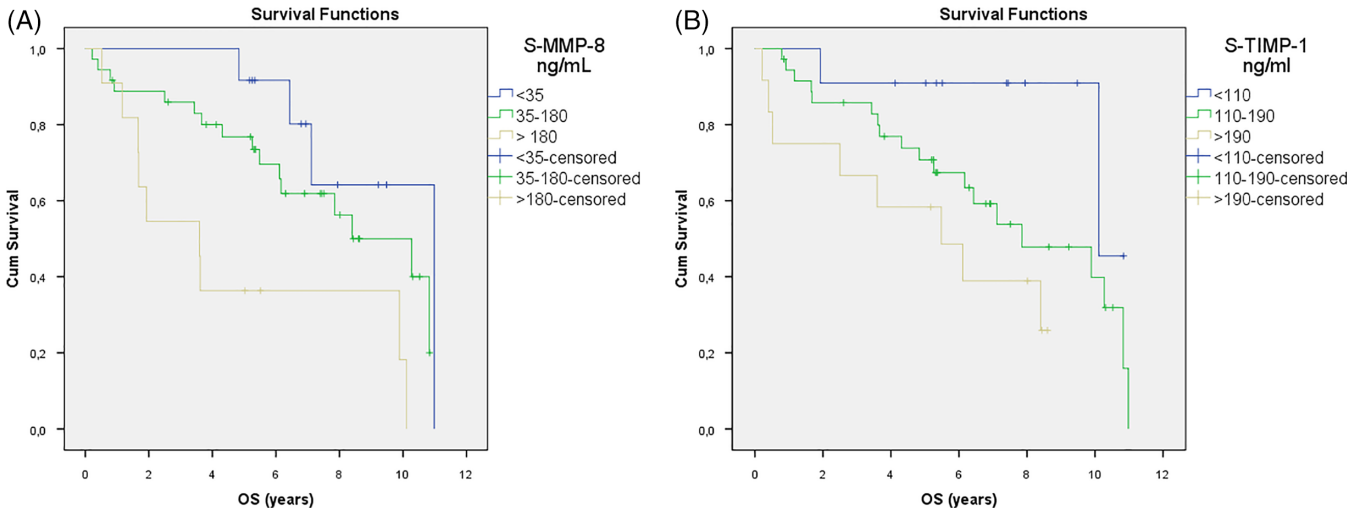

FIGURE 2 A, Overall survival (OS) in 59 patients with laryngeal squamous cell carcinoma (LSCC) according to serum matrix metalloproteinase 8 (S-MMP-8) and (B) tissue inhibitor of metalloproteinase 1 (S-TIMP-1) concentration. Patients were classified according to their serum concentrations so that the highest quintile, 3 middle quintiles, and the lowest quintile comprised 3 groups for comparison [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 5 Cox regression analyses for recurrence-free survival in patients with LSCC

Covariates	HR	95% CI	*P*-value
Age (y)	1.02	0.96-1.09	.50
Sex	1.10	0.22-5.40	.90
Stage	2.21	0.63-7.82	.21
Log_{10}MMP-8	1.27	0.61-2.68	.52
Log_{10}TIMP-1	6.93	1.00-48.17	.05

Abbreviations: CI, confidence interval; HR, hazard ratio; log_{10} MMP-8, log_{10} transformed serum matrix metalloproteinase 8; log_{10} TIMP-1, log_{10} transformed serum tissue inhibitor of metalloproteinase 1; LSCC, laryngeal squamous cell carcinoma.

S-TIMP-1 and DSS was only slightly beyond significance (hazard ratio 12.0, 95% confidence interval 0.95-152.2, $P = .05$). Log_{10} transformed values of S-MMP-8 or MMP-8/TIMP-1 molar ratio did not correlate with any of the survival parameters in multivariate analyses.

4 | DISCUSSION

Compared to LSCC, S-MMP-8 levels in RRP were low. In many inflammatory conditions, MMP-8 expression is upregulated. Although RRP is caused by HPV infection, papillomas are typically small lesions restricted to the vocal fold epithelium. Weak systemic inflammatory response may explain why S-MMP-8 levels in RRP remain low. Interestingly, the patients who later developed LSCC had significantly higher S-MMP-8 levels in comparison to patients with RRP without malignancy. Thus, enhanced systemic MMP-8 response in RRP may predict malignant transformation. Laryngeal papillomas undergoing malignant transformation may cause stronger host responses and production of MMP-8. Premalignant dysplastic lesions of the larynx are often accompanied by a varying degree of mucosal inflammation. Elevated S-MMP-8 levels in patients with RRP developing malignancy may have been associated with laryngeal inflammation. Although this cannot be confirmed in this retrospective material, the predictive role of MMP-8 in chronic laryngitis and precancerous laryngeal lesions is an interesting target for future research. Traditional risk factors for LSCC, smoking, and alcohol consumption are also risk factors for chronic laryngitis, which may proceed to cancer. Lauhio et al. found that smoking can increase S-MMP-8 concentration.[9] In our study, smoking history was reported in patients with LSCC but not in patients with RRP. Smoking was not connected to MMP-8 and TIMP-1 serum levels in patients with LSCC (data not shown).

Malignant transformation has different features among patients with adult-onset RRP and juvenile-onset RRP. In juvenile-onset disease, the patients who develop malignancy are on average younger when diagnosed with RRP. They are seen with an extremely aggressive disease where tracheal or bronchial spread of papillomas is followed by squamous cell carcinoma of the lung. In patients with adult-onset RRP, premalignant lesions are typically limited to the larynx. Some patients have characteristics resembling chronic laryngitis with epithelial hyperplasia and hyperkeratosis. In adult-onset RRP, patients with malignant transformation are on average older when diagnosed with RRP, and they typically develop LSCC. Inflammation and smoking may be cofactors in the process of carcinogenesis, and the exact role of low-risk HPV infection is yet unclear.[10]

One limitation of our study is that the number of patients with RRP with malignant transformation is rather small. A multicenter setting with a larger number of serum samples would give power to our findings. Although RRP is a rare disease, frequently recurring papillomas in a laryngological practice are common. Biomarkers predicting malignant transformation would be useful especially in patients with RRP with long history of smoking, several recurrences, and persisting dysplasia.

Our study agrees with the previous studies in that elevated S-MMP-8 levels are associated with an advanced disease stage.[1,11] For MMP-8, both tumor protective and tumor promoting roles have been suggested.[12–14] MMP-8 plays a central role in inflammation; it is expressed not only by neutrophils but additionally by macrophages, monocytes, T cells, plasma cells, fibroblasts, and epithelial cells.[15] In colorectal cancer, high S-MMP-8 value was associated with stage IV disease and tumor necrosis.[16] Elevated S-MMP-8 levels in our patients with stage III-IV disease may also be associated with tumor necrosis or inflammatory response induced by greater tumor volume. In our study, even patients with small T1 tumors seen with significantly higher S-MMP-8 levels than patients with benign laryngeal papillomas. Serum biomarkers that could indicate the presence of early stage tumors should be evaluated in upcoming studies, considering their potential for cancer screening and early detection of cancer recurrence.

We show that elevated S-TIMP-1 level can predict poor OS in LSCC. Previous studies reporting association between S-TIMP-1 and OS have included heterogeneous groups of head and neck tumors, minority of them presenting in the larynx.[1,3,4] Several factors unrelated to cancer, such as other chronic diseases, may also affect OS. To our knowledge, this is the first study reporting association between S-TIMP-1 and LSCC recurrence. Ma et al. used immunohistochemistry to analyze expression of TIMP-1 in 109 LSCC specimens. Positive staining was mainly localized in the cytoplasm of laryngeal cancer cells, not in the adjacent normal tissues. Multivariate analyses showed that high TIMP-1 expression and lymph node metastases may serve as independent prognostic factors for OS.[17]

Evidence is strong to conclude that in head and neck cancer, TIMP-1 is associated with unfavorable prognosis. TIMP-1 is a multifunctional protein that can promote cell growth, inhibit apoptosis, and regulate angiogenesis.[18] It may thus promote tumor progression and dissemination by

several molecular mechanisms. Because etiology and prognosis of head and neck cancer differs considerably between anatomical subsites, future studies should more specifically report the expression of TIMP-1 in primary tumors by subsite, in adjacent nontumorous tissue, and in peripheral blood.

In univariate analyses, also high S-MMP-8 was associated with poor OS. However, this is likely to be explained by stage III-IV diseases presenting with elevated S-MMP-8. Nonetheless, it should be kept in mind that in former studies S-MMP-8 concentration has not been connected to head and neck cancer survival.[1,4]

Although our study provides new information about S-MMP-8 and S-TIMP-1 levels' connections to malignant transformation of RRP and LSCC prognosis, more studies are needed to develop new diagnostic and prognostic tool for clinical use.

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